Virology 486 (2015) 209–218

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Transgenic down-regulation of ARGONAUTE2 expression in *Nicotiana benthamiana* interferes with several layers of antiviral defenses

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ARTICLE INFO

Article history:
Received 21 May 2015
Returned to author for revisions
15 September 2015
Accepted 16 September 2015
Available online 9 October 2015

Keywords:
N. benthamiana
AGO
Plant virus
Reduction pathogenicity

ABSTRACT

The present study aimed to analyze the contribution of *Nicotiana benthamiana* ARGONAUTE2 (NbAGO2) to its antiviral response against different viruses. For this purpose, dsRNA hairpin technology was used to reduce *NbAGO2* expression in transgenic plants as verified with RT-PCR. This reduction was specific because the expression of other *NbAGOs* was not affected, and did not cause obvious developmental defects under normal growth conditions. Inoculation of transgenic plants with an otherwise silencing-sensitive GFP-expressing *Tomato bushy stunt virus* (TBSV) variant resulted in high GFP accumulation because antiviral silencing was compromised. These transgenic plants also exhibited accelerated spread and/or enhanced susceptibility and symptoms for TBSV mutants defective for P19 or coat protein expression, other tombusviruses, *Tobacco mosaic virus*, and *Potato virus X*; but not noticeably for *Foxtail mosaic virus*. These findings support the notion that NbAGO2 in *N. benthamiana* can contribute to antiviral defense at different levels.

Introduction

Antiviral RNA silencing is a host RNA-mediated defense mechanism that specifically recognizes and degrades single-stranded viral RNA (Baulcombe, 2004). During replication of RNA viruses, double-stranded (ds) or highly structured single-stranded (ss) RNA accumulates and that triggers the host silencing cascade. DICER-LIKE proteins assisted by dsRNA binding proteins cleave these RNAs into short interfering RNAs (siRNAs) of 21–24 nucleotides. Upon their methylation (Yang et al., 2006) siRNAs are recognized by and programmed into an RNA induced silencing complex (RISC) which targets and specifically cleaves cognate mRNA (Alvarado and Scholthof, 2009). The proposed model for RNA silencing in eukaryotes suggests that members of the ARGONAUTE protein (AGO) family form key catalytic units of RISC, which target RNAs for cleavage or translational repression (Baulcombe, 2004).

In the dicotyledenous plant model *Arabidopsis thaliana*, the functions and developmental regulatory capabilities of its 10 known AGOs have been fairly well characterized (Morel et al., 2002; Vaucheret, 2008; Mallory and Vaucheret, 2010). For example, while AGO4, −6, and −9 carry out transcriptional RNA silencing involving 24-nucleotide small RNAs (Havecker et al., 2010; Zheng et al., 2007; Zilberman et al., 2003); AGOs 1 and −7 are known to be programmed with 21- to 22-nucleotide small RNAs such as miRNAs, ta-siRNAs, or exogenously derived siRNAs, such as those from viruses and transgenes (Baulcombe and Baumberger, 2005; Montgomery et al., 2008; Qi et al., 2005). AGOs 1 and −10 are also required for translational control of other miRNA targets and autoregulation (Brodersen et al., 2008; Mallory et al., 2009). AGO1 is a critical developmental regulator, and *ago1* mutants display multiple phenotypes, most notably tubular shaped leaves that resemble the tentacles of an argonaute squid (hence the name-argonaute) (Bohmert et al., 1998). Up-regulation of *AGO1* mRNA has been observed to be a general response to virus infection (Csorba et al., 2007; Havelda et al., 2008; Zhang et al., 2006), probably as an innate defense mechanism. Accordingly, *ago1* mutants exhibit extreme susceptibility to virus infections (Morel et al., 2002). This and other work (Harvey et al., 2011; Jaubert et al., 2011; Qu et al., 2008; Wang et al., 2011) (and as reviewed (Alvarado and Scholthof, 2009; Ding and Voinnet, 2007)) strongly suggest that even though in Arabidopsis AGO2 and AGO7 may contribute to antiviral silencing, AGO1 is key to establishing

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http://dx.doi.org/10.1016/j.virol.2015.09.008
0042-6822/

an antiviral response, presumably because once programmed with siRNAs it forms the slicer component of RISC to specifically target the homologous viral RNA for degradation.

The functional analysis of AGOs in Arabidopsis was possible in part through the availability of shared genetic resources, including specific gene knock-outs. However, for studying plant–virus interactions Arabidopsis has its limitations because of the relatively few viruses that infect this plant. Instead over many decades plant virologists have preferred to use *Nicotiana benthamiana* as the platform to study plant–virus interactions. However, even though the genome sequence (Bombarely et al., 2012) and transcriptome (Nakasugi et al., 2013) of *N. benthamiana* have recently been characterized there is not yet a library available with gene specific knock-outs. Instead gene knock-down studies on *N. benthamiana* are now routinely performed using *Tobacco rattle virus* (TRV) vectors to induce virus-induced gene silencing (VIGS) of specific host mRNAs (Burch-Smith et al., 2004). Even though the knock-down that is achieved yields incomplete loss-of-function (Orzaez et al., 2006; Pflieger et al., 2008), this may occasionally be advantageous, and importantly, sufficient to observe causal effects.

Despite the numerous advantages of the use of the VIGS approach, skeptics often point to possible limitations. For instance, an often-expressed concern is that VIGS necessitates the infection of a host with a virus (*e.g.*, TRV) that may perturb numerous host functions that in turn may mask or interfere with the manifestation of expected silencing phenotypes. This may become especially problematic when the (TRV)-infected plants are challenged with another virus to study its performance in a background where specific mRNAs are targeted by TRV-mediated VIGS. Even when including "empty-vector" TRV controls, this can lead to unexpected synergistic or antagonistic interactions that can influence observations and conclusions. Also, with VIGS experiments there is the potential influence of variation in experimental conditions, and plant-to-plant variation. When studying the antiviral silencing response there is also a paradoxical situation that one depends on an active VIGS to inactivate silencing components that are necessary for VIGS.

Using TRV-mediated VIGS to reduce expression of individual AGOs in *N. benthamiana*, we recently reported that instead of AGO1 (as in Arabidopsis), the antiviral response in *N. benthamiana* against *Tomato bushy stunt virus* (TBSV) is controlled by an AGO2 (Scholthof et al., 2011) analog (NbAGO2). Partly because of the aforementioned reasons relating to possible issues with VIGS, but importantly also to: (i) address the question whether NbAGO2 is specifically used against viral RNA or also for silencing of endogenous (ds)RNA; (ii) to create a stable platform of plants with the inheritable *NbAGO2*-silenced trait; and, (iii) to permit studies on the involvement of NbAGO2 in developmental processes and antiviral defense, we aimed in the present study to transgenically silence *NbAGO2* in *N. benthamiana* without resorting to VIGS. For this purpose a dsRNA-hairpin approach was employed to effectively trigger transient or transgenic gene silencing of *NbAGO2* in *N. benthamiana*. The results showed that the dsRNA approach effectively and specifically down-regulated *NbAGO2* mRNA expression indicating that NbAGO2 is not involved in dsRNA-mediated silencing. Furthermore, the silencing of *NbAGO2* did not affect development under normal growing conditions, but it prevented an effective silencing response against TBSV-GFP not expressing both CP and P19, and it enhanced the susceptibility towards individual TBSV CP or P19 mutants, and selected other viruses.

Results

Transient silencing of NbAGO2

Agrobacterium cultures that harbor the *NbAGO2* dsRNA hairpin-expressing plasmid (*NbAGO2hp*) were infiltrated into *N. benthamiana* leaves. Ten days post-agroinfiltration with cultures expressing *NbAGO2hp*, the silencing-sensor construct TGdP19 (TBSV-GFP neither expressing CP nor P19 (Shamekova et al., 2013)) was also agroinfiltrated onto the putative *NbAGO2*-silenced leaf as well as on an adjacent leaf to monitor the possible movement of the silencing signal. GFP accumulation was monitored under UV light every 5 days (Fig. 1). Five days post-infiltration with the TGdP19 cultures, clear GFP signal was visible on both *NbAGO2hp* and empty vector infiltrated leaves. In the adjacent leaves that had not been infiltrated with *NbAGO2hp* but only with TGdP19, GFP intensity was comparable. This suggested that at this early time-point antiviral silencing of TGdP19 had not yet manifested itself.

However, at 10 days post-agroinfiltration with TGdP19, no GFP signal was visible on the empty-vector agroinfiltrated plants indicating that antiviral silencing had been activated against the

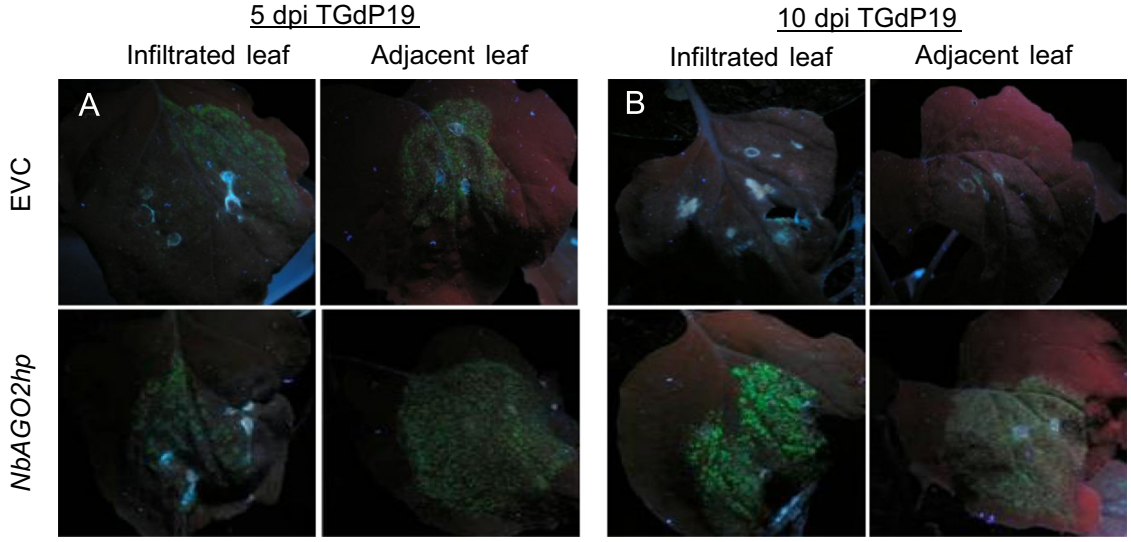

Fig. 1. Transient silencing of *NbAGO2* using the hairpin vector (*NbAGO2hp*). The empty vector control (EVC) or *NbAGO2hp* agroinfiltrated, or non-infiltrated adjacent leaves, were subsequently infected with TGdP19 ten days later, and GFP accumulation was monitored over a 10 day period. The panels show leaves at: A) 5 days post-infiltration (dpi) with TGdP19; B) 10 dpi with TGdP19.

TGdP19 construct (Fig. 1). In contrast, GFP still accumulated abundantly in the *NbAGO2hp*-agroinfiltrated leaves (Fig. 1) supporting the notion that down-regulation of *NbAGO2* compromised the ability of plants to silence TGdP19 (Scholthof et al., 2011). Curiously, the leaves adjacent to the *NbAGO2hp*-infiltrated ones also exhibited green fluorescence at 10 days (Fig. 1), raising the possibility of a limited mobile silencing signal.

From these results we conclude that pre-infiltration of the *NbAGO2hp* construct prevented the ability of the plant to establish an antiviral response against TGdP19, similar to our earlier results obtained with TRV-mediated VIGS (Scholthof et al., 2011). This has two important implications: (1) these results validate the use of the earlier VIGS approach, and (2) the outcome provided experimental justification to conduct further experiments with the *NbAGO2hp* construct to generate stably transformed *N. benthamiana* to address earlier posed questions.

Biological properties of transgenic NbAGO2hp-expressing plants

Based on growth under kanamycin selection, putative transformants were selected and transferred to tissue culture containers (Magenta, Sigma-Aldrich). A leaf portion was used for DNA extraction that was then subjected to PCR screening for the presence of the *NbAGO2hp* containing T-DNA expression cassette (data not shown). Also, using primers designed to selectively amplify the endogenous *N. benthamiana AGO2* transcripts, we confirmed that the levels of *NbAGO2* had been reduced in at least 4 of the 6 selected putative *NbAGO2*-silenced transgenic plant lines, whereas the control plants expressing only the vector did not show the same reduction in transcript levels (Fig. 2; and data not shown). Even though several independent transformant lines (T0 and progeny) exhibiting the most substantial reduction on *NbAGO2* mRNA levels were used at various stages through the study, the results were similar as those presented in the following sections for two separate transgenic lines referred to as A21A and A24C. Because of the similar results obtained with independent transformants, the effects noted in this study are not line- or insertion-site specific, but are due to the silencing of *NbAGO2* caused by expression of *NbAGO2hp*. Semi-qRT PCR analyses of the T1, T2 and T3 generations showed that the *NbAGO2hp* constructs were maintained and expressed in subsequent generations resulting in a reduced level of *NbAGO2* expression, as shown for A21A-T1 in Fig. 2. The expression level of other selected *NbAGO*s was not noticeably affected (Fig. 2), confirming that the *NbAGOhp*-mediated silencing is specifically targeted to *NbAGO2* mRNA.

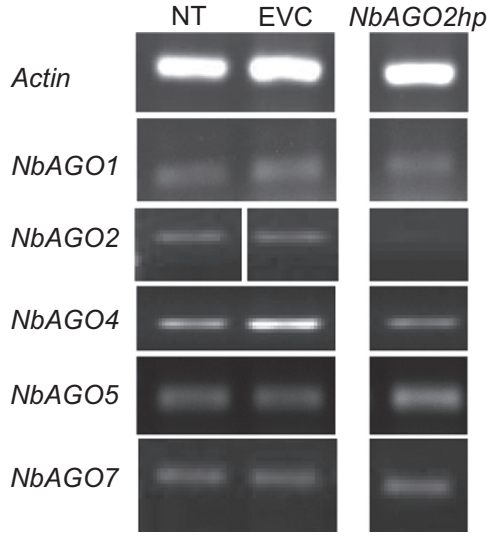

Fig. 2. Hairpin-mediated down-regulation of *NbAGO*2 in transgenic *N. benthamiana*. RT-PCR was used to monitor mRNA accumulation in 4-week old non-transgenic (NT) plants, transgenic empty vector control (EVC) T1 plants, and *NbAGO2hp* transgenic T1 plants. As a control RT-PCR for actin was run for 26 cycles together with *NbAGO1*, *NbAGO2* and *NbAGO4*; *NbAGO5* and *NbAGO7* were run for 35 cycles. Except for *NbAGO2* NT and EVC, PCR bands in each row are from the same gel with intervening lanes removed.

Transformants were transferred to soil and plants grew normally and were brought to flower and seed. This indicated that reduction of NbAGO2 had no effect on normal developmental processes. Curiously, for reasons that are not clear, seeds of the transgenic plants failed to germinate on agar plates in the presence of kanamycin selection at 50 mg/L, therefore, seeds were placed directly in the soil and prior to experiments described in the following sections, plants were screened with PCR to verify their transgenic status. Plants were also periodically screened with RT-PCR or by TGdP19 inoculation (see section below), to verify the maintenance of decline in *NbAGO2* expression.

When seeds were germinated and plants grown under normal growth-chamber conditions, the next generation plants (T1, T2, etc.) developed normally (Fig. 3), just as was observed for the T0 plants described above. However, as a result of serendipitous changes in growing conditions we observed that under sub-optimal conditions the transgenic plantlets were seriously stalled in developing beyond the 3–4th leaf stage (Fig. S1). During the course of study it was noted that these phenotypic effects correlated with the presence of the transgene as verified by PCR. At this point it is not known which external stimulus is responsible for this effect but it does indicate that under stress conditions, NbAGO2 is required at its normal levels early during development.

Silencing against TBSV-GFP not expressing both CP and P19 is substantially impeded in NbAGO2hp-transgenic plants

Agroinfiltration assays with the CP-substitution TBSV-GFP (TG) and TGdP19 constructs on control plants (non-transgenic and empty-vector control plants) revealed that these plants mounted an effective antiviral response since little or no GFP accumulated (Fig. 4) in the absence of the P19 suppressor. This was similar to previous results, just as in the presence of P19 (TG) no silencing occurred (Shamekova et al., 2013). However, in leaves of *NbAGO2hp*-transgenic plants, infections with TGdP19 resulted in an abundant accumulation of GFP almost resembling that of levels obtained with TG based on fluorescence (Fig. 4A) and western blot assays (Fig. 4B). This loss of silencing against TBSV phenotype was maintained into the T2 generation (Fig. S2). In the context of what is known for this system (Scholthof et al., 2011; Shamekova et al., 2013) this supports the conclusion that the antiviral response against TBSV not expressing both P19 and CP is seriously impeded in these transgenic plants.

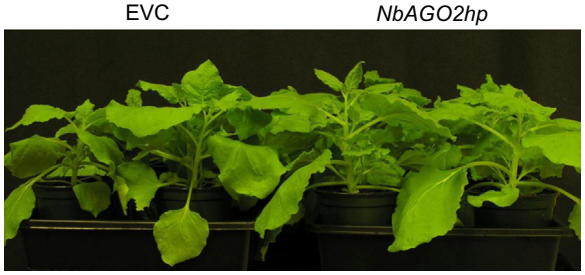

Fig. 3. Phenotype of *NbAGO2hp*-transgenic *N. benthamiana* plants. The two plants on the left-half of the picture are empty vector control (EVC), and the two on the right-half are *NbAGO2hp*-transgenic. Five week old plants were grown at 25–26 °C, 60% humidity and 12 h light at 114 mm/m^2/s.

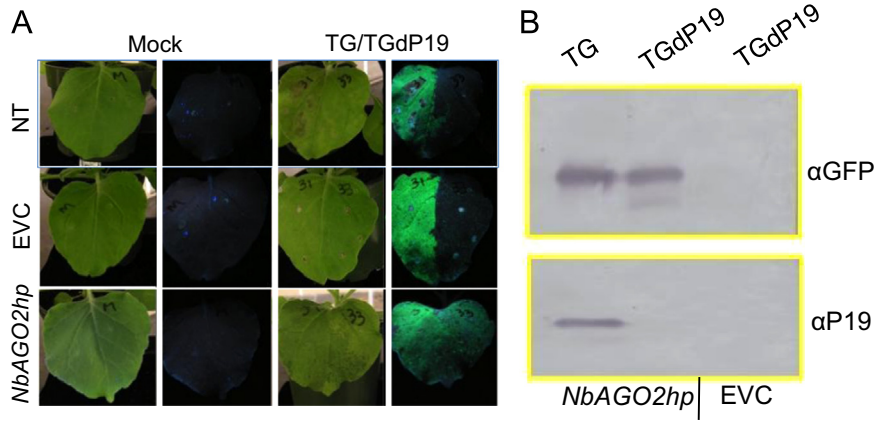

Fig. 4. Silencing activity against TBSV-GFP. (A) Half leaves of 4-week old *N. benthamiana* plants were infiltrated with mock (left panel), or TG (31 on image) and TGdP19 (33 on image) (right panel), and inspected at 7 dpi. The leaves on the left of each panel are shown under ambient light and on the right under UV light. NT, non-transgenic; EVC, empty vector control; *NbAGO2hp*, hairpin transgenic. (B) Western blot showing GFP and P19 expression in 4 week old *N. benthamiana* plants infiltrated with TG and TGdP19, sampled at 8 dpi. Samples are from transgenic *NbAGO2hp* or EVC transgenic plants. Primary antiserum was for GFP (αGFP) or P19 (αP19).

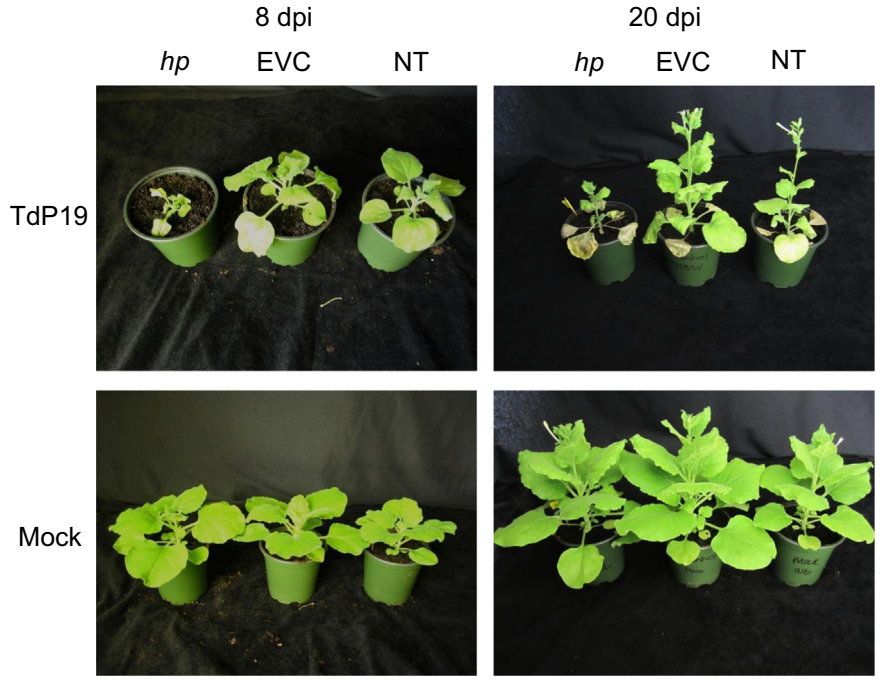

Fig. 5. Symptoms on *N. benthamiana* upon rub-inoculation with TdP19 transcripts. Plants at 8 dpi (left) or 20 dpi (right). *NbAGO2hp* is indicated by *hp*; EVC, empty vector control; NT, non-transgenic.

Augmented systemic infections and symptoms by individual TBSV CP or P19 mutants, and other Tombusviruses

Infections of control *N. benthamiana* plants with a TBSV variant expressing its native CP but not expressing P19 (TdP19), yielded systemic infections with expected (Scholthof, 2006) mild mosaic symptoms. In contrast, TdP19 infections of *NbAGO2hp* transgenic plants resulted in the onset of severe symptoms such as extreme stunting, chlorosis and eventual necrosis (Fig. 5), similar to what was observed when this same TdP19 was used to infect *N. benthamiana* in which *NbAGO2* expression was reduced by TRV-mediated VIGS (Scholthof et al., 2011). Clearly, reduction of NbAGO2 expression not only prevents silencing in inoculated leaves but is permissive for systemic infections and symptoms, somewhat resembling those elicited by infections in presence of P19, albeit much delayed.

In the P19-expressing TG construct the CP gene was replaced with GFP (Shamekova et al., 2013), and normally, infections of *N. benthamiana* with TBSV constructs in which CP is substituted are seriously impeded in the establishment of systemic infections and symptoms (Desvoyes and Scholthof, 2002; Everett et al., 2010; Scholthof et al., 1993; Shamekova et al., 2013). The same was observed in the present study upon infection of control plants with TG (Fig. 6A). Conversely, infections of *NbAGO2hp* transgenic plants with TG resulted in systemic infection accompanied by severe symptoms (Fig. 6A), eventually culminating in a lethal necrosis. Evidently, the reduction in NbAGO2 also affects systemic infections, even in the presence of P19, suggesting that normally CP protects the viral RNA during systemic invasion but this protection is not needed when *NbAGO2* expression is compromised.

To test whether *NbAGO2* down-regulation still affected infections even when P19 and CP are both expressed, the *NbAGO2hp* transgenic plants were inoculated with wild-type TBSV. This resulted in accelerated plant death, compared to symptom progression in control plants (Fig. 6B). We also tested other viruses in the *Tombusvirus* genus of the *Tombusviridae*, including *Cymbidium*

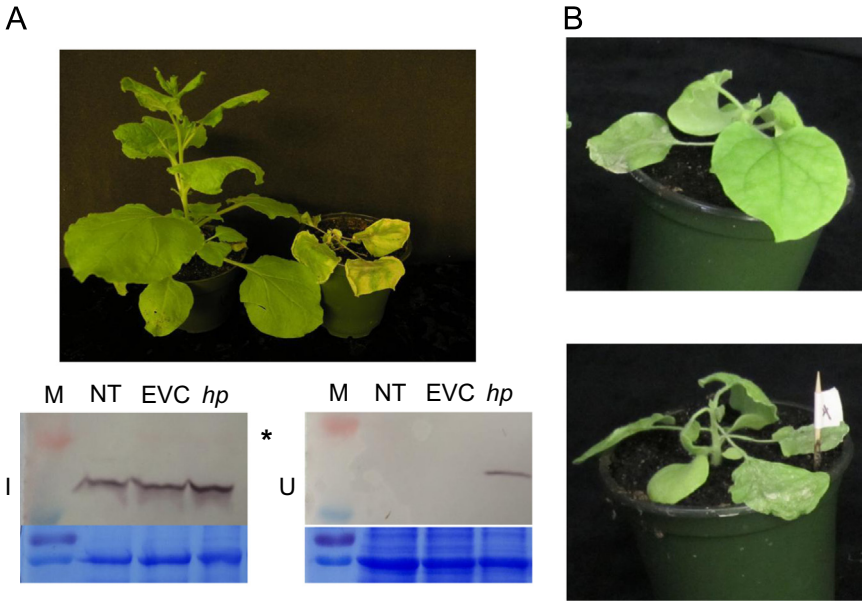

Fig. 6. Symptoms on *NbAGO2hp*-transgenic plants upon inoculation of TBSV in absence or presence of coat protein. (A) Plants photographed 3 weeks after agroinfiltration with TG on non-transgenic (left) or *NbAGO2hp*-transgenic plants (right). The bottom panels show westerns using αP19 for extracts from non-transgenic (NT), EVC, and *NbAGO2hp*-transgenic plants (*hp*), taken from inoculated leaves (I) at 8 dpi, or upper leaves (U) at 13 dpi, on plants inoculated at the 5-week stage. The 25 kDa size marker (M) is indicated (*) and the bottom panels compare loading by staining with Coomassie Brilliant Blue. (B) Plants at 9 dpi with wild-type TBSV transcripts on non-transgenic (top) or *NbAGO2hp*-transgenic plants (bottom). Plants were 4 weeks old upon inoculation. The systemic necrosis is more advanced for *NbAGO2hp* plants compared to control plants where apical necrosis is just developing. Under the conditions used here (ambient light and temperature in laboratory), at 12 dpi *NbAGO2hp* died while the non-transgenic plants remained alive.

ringspot virus (CymRSV), *Carnation Italian ringspot virus* (CIRSV), and *Cucumber necrosis virus* (CNV). In all instances systemic infections on *NbAGO2hp* transgenic plants progressed more rapidly than in control plants and the virus-dependent symptoms were more pronounced (Fig. S3), including accelerated onset of apical necrosis for CNV. Evidently, even when the virus is able to suppress silencing (using P19) and protect its genome during systemic transport (using CP) the effect of down-regulating NbAGO2 remains noticeable, indicative that the *NbAGO2hp* transgenic plants exhibit a general enhanced susceptibility to *Tombusvirus* infection.

NbAGO2 and non-Tombusviruses

To test the effect of NbAGO2 on susceptibility of *N. benthamiana* to viruses other than TBSV, we initially experimented with TRBO-G a robust GFP-expressing *Tobacco mosaic virus* (TMV) variant that does not express CP (Lindbo, 2007) and thus only accumulates in inoculated leaves. We also experimented with a *Foxtail mosaic virus* (FoMV; *Potexvirus*) based vector (Liu and Kearney, 2010a) and a *Sunn-hemp mosaic virus* (SHMV; *Tobamovirus*) vector (Liu and Kearney, 2010b) that express GFP and do not accumulate in absence of an exogenously supplied suppressor, indicating that these viral RNAs are very susceptible to silencing. GFP expression by TRBO-G may have been somewhat elevated in plants in which *NbAGO2* was targeted by TRV-mediated VIGS, compared to controls (Odokonyero, 2012). However, this was not apparent upon inoculation of the *NbAGO2hp* transgenic plants, and likewise the inability of the FoMV and SHMV vectors to establish an infection was not restored upon TRV-mediated VIGS of *NbAGO2* (Odokonyero, 2012), or in *NbAGO2hp* transgenic plants (data not shown).

The above results suggested that NbAGO2 played no clearly discernable role in establishing an infection with the tested TMV, FoMV, and SHMV constructs in inoculated leaves. However, the results with TBSV had taught us that not all noticeable effects may be evident in the inoculated leaves, but rather the effect may surface at the level of systemic infection and symptom development. For this purpose, we infected *NbAGO2hp* transgenic plants with JL24, which is similar to TRBO-G with the difference that it expresses CP which promotes systemic infection (Lindbo, 2007). The results in Fig. 7 show systemic GFP expression in transgenic and control plants, indicating the establishment of systemic infections. However, when compared to controls it is evident that JL24 infections in *NbAGO2hp* transgenic plants exhibited much more severe symptoms to include excessive stunting, wilting of leaves, and ultimately plant death (Fig. 7). Also, time-course studies using western blot analysis to better quantify GFP expression showed that systemic infections with JL24 were reproducibly accelerated by ~24 h in *NbAGO2hp* plants (Fig. 8).

When a *Potato virus X* expressing GFP (PVX-GFP) construct was agroinfiltrated on transgenic and control plants, we detected systemic GFP expression at 10–14 days accompanied by very mild symptoms (Fig. S4). However, compared to control plants, systemic infections (*i.e.*, the appearance of green fluorescence in upper leaves) were noticeably and consistently accelerated by ~24 h in the *NbAGO2hp* transgenic plants (Fig. S4) and symptoms progressed to necrotic spots. This is in agreement with a reported defensive role for AGO2 against PVX in Arabidopsis (Jaubert et al., 2011). Also, at later stages, mature *NbAGO2hp* transgenic plants infected with PVX-GFP exhibited leaf malformations like those normally associated with reduced NbAGO1 accumulation (Jones et al., 2006; Scholthof et al., 2011).

Even though, as mentioned earlier, no defense-negating effects were measurable in *NbAGOhp* transgenic plant leaves inoculated with the SHMV-GFP *Tobamovirus* construct, effects were noted at the systemic infection level for the JL24 derivative of TMV, the type *Tobamovirus* species (Fig. 7). This raised the possibility that a similar scenario could hold for FoMV (*Potexvirus*) for which no effect was seen in leaves inoculated with the FoMV-GFP vector (Odokonyero, 2012), and that possible effects for FoMV should be investigated at the systemic level. For this purpose, transgenic and control plants, were inoculated with wild-type FoMV. However, no obvious differential effects were noted regarding the accumulation in inoculated infected leaves (Fig. S5) while measurements of

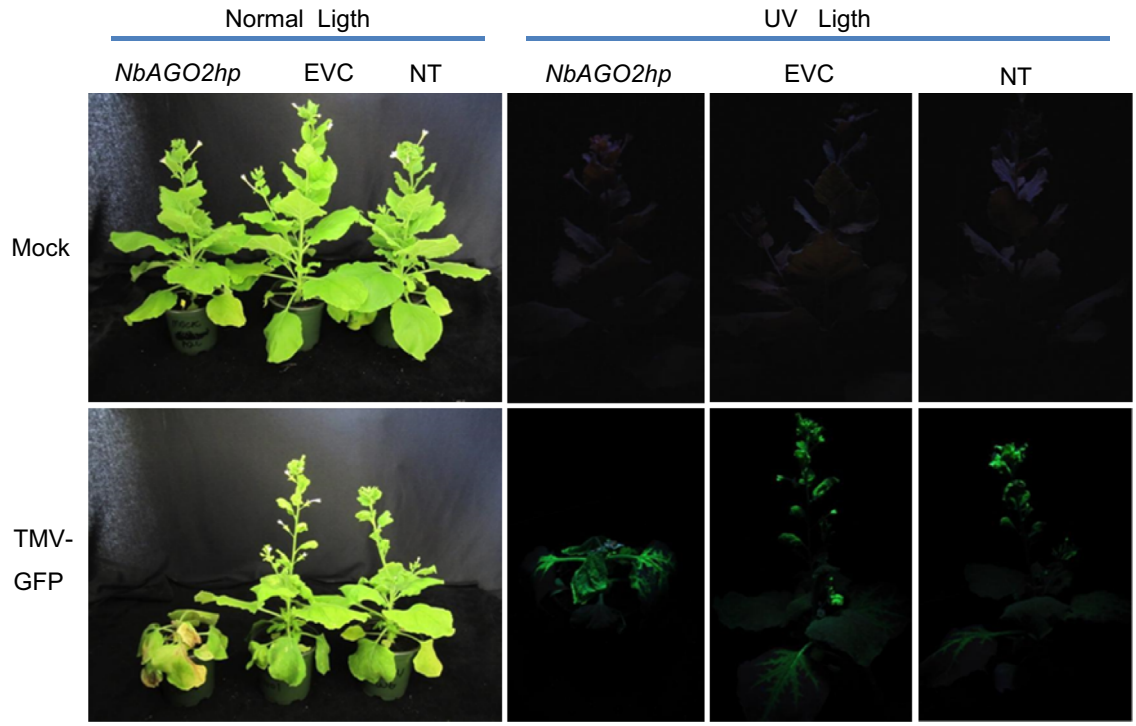

Fig. 7. Infection of plants with TMV-GFP. Infiltration with JL24 (TMV-GFP) was performed on 5 week old plants and images were taken 3.5 weeks later either under normal light or under UV illumination. *NbAGO2hp*, hairpin transgenic; EVC, empty vector control; NT, non-transgenic.

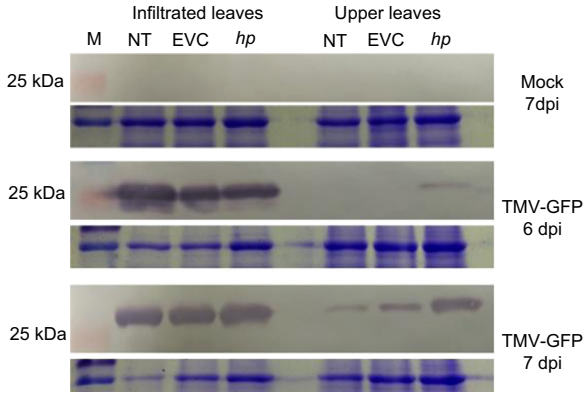

Fig. 8. Western blot for GFP detection upon infection of *N. benthamiana* plants with TMV-GFP. In each of the three panels GFP is shown in upper half while the lower half shows the loading upon staining of the gels with Coomassie brilliant blue. Mock represent plants infiltrated with infiltration buffer alone and protein samples for this treatment were collected at 7 days post-infiltration. pJL24 (TMV-GFP) infiltrated samples were collected at 6 (middle) and 7 (lower) days post-infiltration. NT, non-transgenic; EVC, empty vector control transgenic plants; *hp*, *NbAGO2hp* transgenic plants. The position of the 25 kDa molecular size marker (M) is indicated.

effects on the ineffective systemic invasion were inconclusive. Therefore, NbAGO2-associated effects noted for PVX are not measurably recapitulated for its *Potexvirus* relative FoMV.

Discussion

Transient silencing of NbAGO2

Transient assays with the *NbAGO2hp* or control infiltrated *N. benthamiana* leaves showed that upon agroinfiltration of these same leaves with TGdP19, GFP expression was evident only in *NbAGO2hp* treated leaves. This indicates the persistence of the effects in the host without significantly interfering with normal physiological processes. These observations not only further confirm the antiviral defense role of NbAGO2 against TBSV as previously reported (Scholthof et al., 2011), but also prove the effectiveness of hairpin RNA as a tool for silencing of *N. benthamiana AGO* genes. In fact, the efficiency levels are comparable to the commonly used TRV-virus induced gene silencing systems. Therefore, both techniques yield very comparable results and may remain attractive options if gene knock-out strategies, that are becoming increasingly applied for *N. benthamiana,* for instance by implementation of the CRISPR/Cas9 system (Li et al., 2014; Nekrasov et al., 2013), are not desired.

The results also suggest that a systemic 'hairpin-associated signal' had spread to adjacent leaves not infiltrated with the hairpin construct. RNA silencing has been shown to be non-cell-autonomous, with the capability of being induced locally and then spread to distant sites throughout the plant (Boerjan et al., 1994; Palauqui et al., 1996, 1997; Vaucheret et al., 1997) and propagated by means of some 'mobile signal' (Palauqui and Vaucheret, 1998).

Development of NbAGOhp transgenic plants

The results in Figs. 2 and 4 show that the transgenic dsRNA approach effectively and specifically down-regulated *NbAGO2* mRNA expression in transgenic *N. benthamiana* indicating that NbAGO2 is either not, or only at low levels, involved in transgenic dsRNA-mediated silencing. This supports our earlier observations using VIGS that NbAGO2 was neither involved in the ability of TRV to induce silencing nor in transient dsRNA-mediated silencing (Scholthof et al., 2011).

In earlier experiments no phenotypic effects were noticed when *NbAGO2* expression was down-regulated in *N. benthamiana* plants using TRV-based VIGS that was initiated on ~3 week old plants (Scholthof et al., 2011). Likewise, agroinfiltration with a *NbAGO2hp*-expressing construct in the transient assays did not lead to any noticeable phenotype either (Fig. 1). Similarly, when

NbAGO2hp transgenic plants are grown under normal conditions they are, at the macroscopic level, phenotypically indistinguishable from control plants. Furthermore, the transgenic plants flowered and set seed normally, and for the different generations these seeds were viable and germinated at rates comparable to the wild-type and EVC-transformed plants. These findings suggest that under our conditions *NbAGO2* reduction did not cause irregularities in plant development. Alternatively, if in the future it turns out that *NbAGO2* is required for regulation of plant growth and development, it may be needed only in the trace amounts that are still produced upon its hairpin-mediated down-regulation.

When growth conditions were sub-optimal the development of the *NbAGO2hp* transgenic plants was compromised at early stages. This may be related to the observation that in comparison to the controls, we experienced some recalcitrance in the efficacy of regenerating *NbAGO2hp*-transgenic explants from callus. These phenomenological observations need further experimental scrutiny but they allude to a notion that NbAGO2 may have a supportive role early during development of *N. benthamiana* that surfaces under stress conditions, while at later stages it is no longer needed, or only at low levels, and can then be recruited into the plant's antiviral defense arsenal.

Antiviral effects against P19 and/or CP inactivated viruses versus wild-type versions

Upon agroinfiltration of the TGdP19 construct onto successive generations of *NbAGO2*-down-regulated transgenic plants (*e.g.*, Fig. 4), we observed the accumulation of GFP in leaf tissue confirming the trans-generational persistence of *NbAGO2* gene silencing that protects the otherwise silencing-susceptible TGdP19. Clearly, when the virus is no longer protected from silencing by the P19 suppressor, it is susceptible to the NbAGO2-mediated defense. But, this defense can be disarmed by reducing NbAGO2 accumulation.

In contrast to controls plants, *NbAGO2hp* transgenic plants infected with TdP19 (expressing CP, but not P19) exhibited severe and often lethal systemic symptoms, that are normally associated with P19 expression. This suggests that part of the severe symptoms associated with TBSV on *N. benthamiana* are attributable to interference with antiviral silencing to protect the virus, that can either be achieved naturally by P19-mediated suppression (Scholthof, 2006) or experimentally by down-regulation of *NbAGO2*.

Infections of *NbAGO2hp* transgenic plants with TG (expressing P19, but not CP) resulted in severe systemic symptoms (Fig. 5) eventually leading to a lethal necrosis. Such severe systemic invasions are normally not seen for TG (Shamekova et al., 2013) or other CP-defective TBSV mutants in *N. benthamiana* (Desvoyes and Scholthof, 2002; Qiu et al., 2002;Qiu and Scholthof, 2001; Scholthof et al., 1993). These results suggest that the reduction of NbAGO2 in *NbAGO2hp* plants affects systemic infections of TBSV without the CP. It is plausible that under normal conditions the CP protects the virus against NbAGO2-associated antiviral defenses during systemic invasion, but when *NbAGO2* expression is compromised this protection by CP is less important, resulting in a severe systemic infection even in absence of CP.

Even though wild-type TBSV infections are accelerated in *NbAGO2hp* transgenic plants, the effect is much less extensive than as discussed above for the P19 and CP mutants, which suggest that other biological studies with wild-type versus mutant viruses could also easily lead to different interpretations. For instance, it is known that various proteins of tombusviruses can cause a HR upon infection but even without a (functionally active) elicitor of the necrotic response the defense in the form of lesions or ringspots can remain operative (Angel et al., 2011; Angel and Schoelz, 2013; Chu et al., 1999, 2000). Specifically, when comparing results of defense responses on resistant *N. tabacum* (a necrotic local lesion host for TBSV, with P19 as elicitor) using TGdP19 not expressing CP (Sansregret et al., 2013) versus TdP19 expressing CP (Scholthof et al., 1995) important differences surface. The authors of the former study are reporting a finding that there is an extreme resistance (ER) response when using TGdP19, but in the much earlier study with TdP19 this ER was not noted since lesions still formed. Also, the two systems yield different interpretations about the possible involvement of siRNA binding for the resistance response (Hsieh et al., 2009; Sansregret et al., 2013). These findings strongly suggest that the lack of CP influences the outcome of the studies, and it seems to seriously hinder TBSV invasion of *N. tabacum*, which apparently manifests itself as ER.

Our findings also show that while NbAGO2 is clearly involved in anti-TBSV defense, its antiviral activity may be more widespread than previously thought. GFP-expressing *Tobamovirus* and *Potexvirus* variants that are very sensitive to silencing, are not able to infect *NbAGO2hp* transgenic plants. However, the wild-type based *Tobamovirus* TMV-GFP did exhibit an enhanced ability to infect *NbAGO2h* transgenic plants while this was not evident for the *Potexvirus* FoMV. Yet, NbAGO2 down-regulated plants show enhanced susceptibility to systemic infection with PVX-GFP, and also for several tombusviruses, and the same trend was observed upon infections with *Brome mosaic virus* (not shown). Collectively, the results show that depending on the virus and whether the virus construct expresses a suppressor and/or a coat protein, the contribution of NbAGO2 to the antiviral response may surface in inoculated leaves, during systemic transport, or in the onset and severity of systemic symptoms.

Roles of different NbAGOs in N. benthamiana

Several studies have shown that in Arabidopsis AGO1 plays a vital antiviral defense role with different levels of contribution by AGO2 and AGO7 (Alvarado and Scholthof, 2012; Carbonell et al., 2012; Harvey et al., 2011; Jaubert et al., 2011; Qu et al., 2008; Wang et al., 2011; Zhang et al., 2012) and more recently shown for AGO5 (Brosseau and Moffett, 2015). Much less is known regarding the antiviral activities of AGOs in *N. benthamiana,* but it was established that NbAGO4 has an effect on translation during a viral resistance response (Bhattacharjee et al., 2009). Furthermore, although NbAGO1 did not contribute to silencing of TBSV (Scholthof et al., 2011), it does have a novel translation-mediated defensive effect against *Tomato ringspot virus* infection in *N. benthamiana* (Ghoshal and Sanfaçon, 2014; Karran and Sanfacon, 2014). The present study with transient and transgenic expression of *NbAGO2* hairpin RNA, firmly confirms that NbAGO2 is necessary for antiviral silencing against TBSV. Considering that Arabidopsis AGO2 has 'slicing' activity (Carbonell et al., 2012), it is possible that NbAGO2 represents a catalytic component of the enzymatically active RISC that was previously isolated from TBSV (P19 mutant) infected plants (Omarov et al., 2007). Collectively, the reports thus far seem to support the premise that depending on the circumstances, several AGOs in plants can be deployed to combat virus infections.

Conclusion

This study shows that AGO2 in *N. benthamiana* contributes to antiviral defenses in inoculated leaves and upon systemic infection. The antiviral effect of NbAGO2 towards TBSV is strongly evident in leaves inoculated with a GFP-encoding mutant not expressing both P19 and CP. Upon inoculation of individual CP or P19 defective mutants onto NbAGO2 down-regulated *N. benthamiana* plants, systemic infections and symptoms develop that do not occur in control plants. Even upon infection with wild-type TBSV, other tombusviruses, or unrelated TMV and PVX, down-

regulation of NbAGO2 accelerates infection and associated symptom induction. Therefore, we conclude that when NbAGO2 is expressed at normal levels in *N. benthamiana*, it serves as a reducer of viral pathogenicity at different stages during infection. Down-regulating the host-encoded pathogenicity reducer increases the invasive ability of the virus, especially for otherwise silencing-sensitive disarmed viruses inactivated for expression of a 'protective' suppressor and/or coat protein.

Materials and methods

The NbAGO2 hairpin RNAi vector

Gateway recombination cloning techniques (Life Technologies, Carlsbad, CA) were used to clone the dsRNA expressing gene segment. In the first step, template-specific primers containing the *att*B sites plus the gene specific primers were used in a 10-cycle PCR run to amplify the target *NbAGO2* fragment from the original TRV construct (Scholthof et al., 2011). In the second step, 10 μL of the reaction mixture from the first PCR was used as the DNA template and *att*B adapter-specific primers were used to amplify the full *att*B PCR product. Approximately 10 μL of the resulting products from the second PCR were electrophoresed through a 1% agarose gel to confirm amplification of the correct size fragment. The PCR products were purified using Qiagen's QIAquick gel extraction kit (QIAGEN, Valencia, CA) and eluted in 40 μL of TE (10 mM Tris–Cl pH 8 and 1 mM EDTA). The BP clonase reaction was carried out by mixing 150 ng of the vector, 25 ng of purified *aatB* PCR product and 1 μL of BP clonase enzyme (Life Technologies, Carlsbad, CA). The mixture was gently agitated and incubated at 25 °C overnight to simultaneously recombine the identical fragments in different orientations into a specially designed T-DNA vector (pHellsgate2) (Helliwell and Waterhouse, 2003; Smith et al., 2000; Wesley et al., 2001) to yield a self-complementary gene construct encoding an inverted repeat of a fragment of the *NbAGO2* gene sequence separated by a PDK intron which upon transcription provides a source of the dsRNA trigger needed to initiate the PTGS process (Helliwell and Waterhouse, 2003; Smith et al., 2000; Wesley et al., 2001).

Two microliters of the BP clonase reactions were electroporated into DH10β strain of *Escherichia coli* for screening. After culturing, plasmid DNA was isolated using Qiagen's QIAprep Spin Miniprep Kit (QIAGEN Valencia, USA) following the manufacturer's suggested protocol. Restriction digestions were carried out separately using *Xba*I and *Xho*I enzymes (New England Biolabs, Ipswich, MA) in order to ensure that the gene fragment had been inserted at both sides of the PDK intron and a PCR reaction to confirm that the gene insertions were correct. DNA of the resulting hairpin construct (*NbAGO2hp*) DNA was also sequenced to confirm that no undesirable spontaneous mutations were present.

Agroinfiltrations and virus inoculations

The *NbAGO2hp* construct was electroporated into GV3101 (also known as pMP90RK) strain of *Agrobacterium tumafaciens*. A control 'empty' vector (with a serendipitously isolated mutated hence non-functional *ccdB* gene) was also transformed into *Agrobacterium* to serve as a negative empty vector cassette (EVC) control. *Agrobacterium* cultures containing the silencing construct (*NbAGO2hp*) and controls, were grown overnight (16–20 h) under constant shaking (200 rpm) at 28 °C in 5 mL Luria broth (LB) media with kanamycin at 50 mg/L. *Agrobacterium* cells were collected by centrifugation at 3500 rpm for 20 min and the cells resuspended in infiltration media (10 mM $MgCl_2$) to a final OD 600 of 0.5. The abaxial sides of leaves of five-week old plants were agroinfiltrated and maintained at 25 °C for 10 days.

Plasmids carrying TG and TGdP19 (Shamekova et al., 2013), PVX-GFP (Peart et al., 2002) and JL24 (Lindbo, 2007) were also cultured in *Agrobacterium* GV3101 and agroinfiltrated (OD_{600}: 0.5) onto *N. benthamiana,* as above. In the case of wtTBSV, TBSVdp19 (Qiu and Scholthof, 2001), and the other tombusviruses *in vitro* transcription reactions were conducted on *Sma*I digested plasmids with T7 RNA polymerase (Fermentas Life Sciences). Transcripts were mixed in RNA inoculation buffer (0.05 M Potassium phosphate monobasic, 50 mM Glycine pH 9.0, 1% bentonite, and 1% Celite) and inoculated onto *N. benthamiana* plants, as described previously (Scholthof et al., 1993). FoMV-infected plant sap (from laboratory virus collection repository, (Mandadi et al., 2014)) was used as the source of inoculum in the present experiments.

Imaging of GFP fluorescence was performed as previously described (Everett et al., 2010).

Protein extraction and western blot assays

For each treatment 50 mg of plant tissue was collected, ground in liquid nitrogen and 300 μL of 5 × Laemmli sodium dodecyl sulfate (SDS) protein extraction buffer was added. The samples were denatured in boiling water for 5 min and 25 μL loaded onto a 10% polyacrylamide-SDS gel and initially run at 60 V for 1 h and then 2.5 h at 110 V. The proteins were transferred to a nitrocellulose membrane (Biorad, CA) in transfer buffer (25 mM Tris, 192 mM glycine) at 260 mAmp for 90 min. Then, the membrane was blocked in 5% non-fat milk in a Tris-buffered saline solution (TBS) (0.2 M NaCl, 50 mM Tris, pH 7.4) for 1 h and incubated with the primary antibody (anti-GFP (B-2) mouse monoclonal (Santa Cruz Biothechnology) at 1:10,000 dilution, or rabbit P19 antibodies and anti-FoMVH93 (laboratory stock) at 1:3000 dilution) for 1 h. After incubation, three washes for 5 min each were performed. The secondary antibodies were anti-mouse or anti-rabbit alkaline phosphatase (Sigma) at 1:10,000 dilution. These were incubated for another hour, then 4 washes were performed with TBS and 1 wash with TBST (0.05% Tween) for 5 min. Finally, each membrane was incubated for colorimetric detection by using 1 × alkaline phosphatase buffer (100 mM Tris, pH 9.5, 1 M $NaCl_2$, and 0.5 M $MgCl_2$ 6(H_2O)), 33 μL of NBT (250 mg/ml) and 66 μL of BCIP (100 mg/ml).

N. benthamiana transformation and regeneration

Plasmid DNA was electro-transformed into *Agrobacterium* strains GV3101 or LBA4404, cultured in 25 mL LB+50 ng/mL kanamycin, and grown at 28C with vigorous shaking (250 rpm) for 16–20 h. The cultures were then each transferred into 50 mL tubes and centrifuged at RT for 5 min at 5000 rpm. The resulting pellet was resuspended in liquid MS medium to a final concentration between 0.3 and 0.6 OD600.

Leaves of young *N. benthamiana* plants were cut into ~1 cm × 1 cm disks with a cork borer, and placed in a small amount of liquid MS medium. The transformed *Agrobacterium* was added onto the cut leaf pieces for about 15 min after which the excess bacteria solution was blotted off using pieces of filter paper. The leaf pieces were transferred to a co-culture medium (MS-zero supplemented with 6-BA (benzyl adenine) at 1.0 mg/L, and NAA (1-Naphthaleneacetic acid) at 0.1 mg/L, and the petri dishes were placed in a dark incubator at 28 °C. Upon visual inspection of *Agrobacterium* growth on the plates (about 1–2 days), disks were transferred to a fresh selection medium (MS0+6-BA 1.0 mg/L+NAA 0.1 mg/L+ Kanamycin 100 mg/L+Carbenicillin 300 mg/L). When Agrobacterium growth was excessive, explants were rinsed three times in sterile water and filter paper was used to blot off the excess water before placing them on fresh selection medium. The

plates were placed in a 28 °C incubator with 16/8-h light/dark conditions.

One week later, explants were again transferred to fresh selection medium plates (MS0+6- BA 1.0 mg/L+NAA 0.1 mg/L+Kan 100 mg/L+Carbenicillin 200 mg/L). The explants were then routinely transferred to fresh selection medium plates every 16–20 days when the shoots were about 1.5 cm long. A clean cut was made at the base of each shoot to ensure there was no callus attached, and transferred to rooting medium (MS0+NAA 0.1 mg/L+Kan 100 mg/L+Carbenicillin 200 mg/L). The putative transgenic plants were kept in a growth chamber at 25 °C with 16−8 light–dark hours and 60% humidity. After transfer to soil, plants were grown in growth chambers under similar conditions except during later stages of the study when conditions were changed to 12–12 light–dark hours.

RNA and DNA extractions, and (RT)-PCR conditions

For total RNA extraction, 0.1 g of plant tissue was collected and ground using a mortar and pestle in 750 µl extraction buffer (80 mM Tris pH 8.2, 40 mM LiCl, 1.96 mM EDTA, 0.44% SDS, 78.57 mM NaAc pH 4.0, 0.44 M phenol, 87 mM chloroform and 0.44 mM β-mercaptoethanol) and centrifuged at 12,000 rpm for 6 min at room temperature. The supernatant was collected and mixed in a 1:1 ratio with a phenol:chloroform:isoamyl-alcohol (25:24:1) mixture. The mixture was further centrifuged for 6 min and the supernatant was collected, mixed with chloroform at 1:1 ratio and centrifuged at 12,000 rpm for 6 min. The supernatant was collected, mixed with 1/3 volume of 8 M LiCl and precipitated overnight at −20 °C. The RNA was pelleted by centrifugation at 4 °C at 13,000 rpm for 20 min, and the pellet washed in 70% ethanol and resuspended in 30 µl DEPC-treated water. The obtained RNA was treated with Turbo DNA-free kit (Applied Biosystems) prior to cDNA synthesis using oligo-d(T) primers and M-MLV reverse transcriptase (Invitrogen).

To extract plant genomic DNA, approximately 200 mg of leaf tissue was thoroughly macerated in 750 µL of extraction buffer (100 mM Tris pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl, 10 mM β-mercaptoethanol) using a mortar and pestle. Then, 35 µL of a 20% SDS solution was added and incubated in a 65 °C heat block for 5 min followed by addition of 130 µl of potassium acetate, gentle mixing and incubation on ice for 5 min. The resulting debris was then pelleted by centrifuging at 15,000*g* for 10 min at room temperature. The supernatant was extracted and 750 µL of isopropyl alcohol followed by 75 µL of 3 M NaAc pH 5.2, were added, the tubes gently inverted to mix and at incubated in a −20 °C for at least 1 h. DNA was then pelleted by centrifugation at 15,000*g*, the supernatant was discarded and 70% ethanol was used to wash the resulting pellet. Excess ethanol was evaporated in a spin-vacuum centrifuge for approximately 30 min. The DNA was then resuspended in 30 µL of TE containing 20 µg/mL RNase. The mixture was incubated at 37 °C for 15 min and then centrifuged at 15,000*g* for 5 min and diluted to a final volume of 60 µL for immediate use or storage at −20 °C.

PCR was carried out to verify the presence of the *NbAGO2hp* construct as well as transcript levels of *NbAGO2*. Primary transformants were screened by regular DNA extraction followed by conventional PCR and first and second generation transgenic plants (T1 and T2) were screened with Terra PCR Direct Polymerase kit (Takara Biotech) using primers for native *NbAGO2* sequence 5′-GAGCACTTGGCTGAACATGA-3′ and vector sequences 5′-CTTGTAGTTTTATTAACTTCT-3′. Primers for amplification of cDNA to measure relative transcript levels are presented in Table S1.

Acknowledgments

We thank John Lindbo for TRBO-G and JL24, Chris Kearney for the FoMV and SHMV GFP expressing vectors (and Will Cody for testing these on the transgenic plants), Peter Moffett and Chantal Brosseau for PVX-GFP and suggestions, Luisa Rubino for CymRSV and CIRSV, and D'Ann Rochon for CNV, Peter Waterhouse for the pHellsgate vector, and the Department of Biology at Texas A&M University for generously allowing us to use their real-time PCR facility. We are grateful for the helpful comments by Karen-Beth G. Scholthof and her research group at various stages through the study and during manuscript preparation, and for providing FoMV and BMV virus inocula. The work was possible through funding from Texas AgriLife Research (TEX08387), USDA/CSREES-NRI-CGP2006-35319-17211, and awards from USDA-NIFA-AFRI2012-67013-19397 and 2015-67013-22916.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:http://dx.doi.org/10.1016/j.virol.2015.09.008.

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